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Structural Difference between α-Paramyosin and β-Paramyosin of *Mercenaria mercenaria*[†]

Anthony T. Yeung and Robert W. Cowgill*

ABSTRACT: A method is described for extraction of α-paramyosin in amounts comparable to that formerly attained for β-paramyosin (15–25 mg/g of muscle). A modification of the procedure for sodium dodecyl sulfate gel electrophoresis is described that permits the separation on coelectrophoresis of α-paramyosin (207 000 daltons) and β-paramyosin (200 000 daltons). The α- and β-paramyosins also can be distinguished by gel electrophoresis at pH 2.3 and by differences in solubility in the region of 0.2–0.4 ionic strength at neutral pH. Evidence is presented that the segment lost from α-paramyosin during degradation to β-paramyosin came from the C-terminal end. This evidence is based on determinations of N- and C-terminal amino acids and on the size of segments obtained after chemical cleavage at the sites of Cys residues. It has been observed earlier that the solubility characteristics of β-paramyosin at neutral pH are determined by the C-terminal one-third of the molecule and the present results indicate that the additional small segment of about 3.5% of the total mass that is present in the C-terminal end of α-paramyosin accounts for the marked difference in solubility of the two forms.

Paramyosin is a major protein in the thick filaments of molluscan adductor muscles. The protein is a double α-helical rod-like molecule about 1255 Å long and 20 Å in diameter (Lowey et al., 1963; Cohen et al., 1971). Stafford and Yphantis (1972) have reported that paramyosin as extracted from adductor muscles of the clam *Mercenaria mercenaria* by the conventional ethanol-denaturation procedure of Johnson et al. (1959) is in fact a proteolytically degraded form. By including 0.01 M EDTA[1] in all their buffers, Stafford and Yphantis were able to extract a form of paramyosin slightly larger in molecular weight which they called α-paramyosin of 210 000. The conventional form of 200 000 was termed β-paramyosin. The presence of a smaller form termed γ-paramyosin of 188 000 also was observed after some extractive procedures and this was presumed to be a product of more extensive degradation of α-paramyosin.

α-Paramyosin is probably the form that occurs in muscle filaments, and it may function there by virtue of having regions of different stability (Riddiford, 1966; Olander, 1971; Cowgill, 1972, 1974; Halsey and Harrington, 1973). Also, the solubility of paramyosin at physiological conditions may be important for self-aggregation and thus for assembly of the thick filaments of muscle. The extra segment of α-paramyosin may be of importance for this purpose because it has been shown that different segments of β-paramyosin possess different solubility characteristics (Cowgill, 1975) and the solubility of α-paramyosin differs from that of β-paramyosin (Merrick and Johnson, 1974). It seemed important to locate the segment on α-paramyosin that was removed upon degradation and to determine properties such as solubility that could be attributed to that small segment. Therefore it is the purpose of this paper

[1] Abbreviations are: PTC-1, paramyosin trypsin resistant core; CCF-1 and CCF-2 are the two segments released from paramyosin by cleavage at the site of Cys residues; Nbs₂, Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

to describe a new method for extraction of α-paramyosin in higher yield than attained formerly, to present evidence that the segment lost from α-paramyosin upon conversion to β-paramyosin came from the C-terminal end, and to demonstrate the significant effect of this small segment upon the solubility of the molecule.

Materials and Methods

Materials

The enzymes and other proteins for molecular weight standards, the standard dansyl amino acids, and other organic chemicals were all of the highest purity available. Guanidine hydrochloride was of the Ultra-Pure grade from Schwarz/Mann Research Laboratory, and fluorescamine was purchased from Roche Diagnostics. Hog kidney leucine aminopeptidase and carboxypeptidase A (diisopropyl fluorophosphate treated) were purchased from Worthington Biochemicals. Myosin from rabbit skeletal muscle was prepared in this laboratory by the method of Szent-Györgyi (1951).

Methods for the Extraction and Treatment of Paramyosin

A. *Isolation of Paramyosin.* Clams of the *Mercenaria mercenaria* species were obtained live from the coast of North Carolina and Delaware. The whole adductor muscles were excised, washed in 0.01 M EDTA solution at pH 7 on ice, and stored at −20 °C. The following procedures describe the isolation from this muscle of α-paramyosin in the presence of 0.01 M EDTA to prevent proteolysis and the isolation of β-paramyosin without EDTA so that proteolytic degradation occurred.

α-Paramyosin was isolated initially by the ethanol-denaturation procedure of Johnson et al. (1959) but with 0.01 M EDTA included in all buffers as recommended by Stafford and Yphantis (1972); however, the yield of pure α-paramyosin by this procedure was only about 5 mg/g of whole muscle. Because of the very poor yield, a modification of the "selective-extraction" procedure of Szent-Györgyi et al. (1971) was employed to circumvent the ethanol-denaturation step which appears to render α-paramyosin insoluble. (Dialysis was at 4 °C and all other steps were at 0 °C.) Whole adductor muscles were cut into small pieces and homogenized for 30 s in a Waring blender with 5 volumes of 0.1 M KCl-0.01 M EDTA, pH 7. Another 5 volumes of the same buffer was used to rinse the blender and combined with the homogenate which was stored on ice for 2 h with occasional stirring. The homogenate then was centrifuged for 15 min at 4300g and the pellet was washed three times, each with 7 volumes of the same buffer. The last centrifugation was for 20 min at 12 000g. The pellet of washed filaments was extracted three times for 15 min each with 3-volume portions of 0.4 M KCl-0.05 M KPO$_4$-0.01 M EDTA (pH 6) to remove actomyosin. Care was taken to maintain the pH at 6 to avoid the removal of paramyosin, which will occur at higher pH's. The extracts were centrifuged at 12 000g and the supernatant solutions were discarded. The residual pellet was extracted for α-paramyosin by two 30-min extractions, each with 4 volumes of 1.0 M KCl-0.05 M KPO$_4$-0.01 M EDTA (pH 7.5). The extracts which contained the paramyosin were centrifuged at 5900g for 20 min, and then the supernatant solutions were combined and centrifuged again for 25 min at 27 000g. The pellet from the latter centrifugation was discarded, and the supernatant solution was dialyzed against a large volume of 0.4 M KCl-0.05 M KPO$_4$-0.01 M EDTA (pH 7.5). The dialyzed solution was centrifuged for 20 min at 12 000g, and any small pellet was discarded. The su-

pernatant solution was dialyzed twice against 1-1. amounts of 0.4 M KCl-0.05 M KPO$_4$-0.01 M EDTA (pH 6) until the final pH was less than 6.2. The paramyosin crystalline suspension was centrifuged for 20 min at 5900g, and the supernatant solution was discarded. The pellet of paramyosin was dispersed in 6 volumes of 0.4 M NaCl-0.05 M NaPO$_4$-0.01 M EDTA (pH 7.5) and, without removal of insoluble material which consisted in part of the slowly dissolving paramyosin, it was dialyzed against a large volume of 0.4 M NaCl-0.05 M NaPO$_4$-0.01 M EDTA (pH 7.5). Then residual insoluble proteins were removed by centrifugation. Additional crystallizations at pH 6 were performed by the same procedure as above.

β-Paramyosin was initially extracted by the ethanol-denaturation procedure of Johnson et al. (1959) with minor modifications by Cowgill (1972). Later, to facilitate comparison of yields, the selective-extraction procedure just described was employed except that EDTA was omitted from the solutions so that proteolytic degradation occurred. Also, the solution for crystallization of β-paramyosin contained only 0.05 M NaPO$_4$ at pH 6 because β-paramyosin is more soluble (about 0.8 mg/ml) at the higher ionic strength of the buffer used for α-paramyosin crystallization.

B. *Formation of α-Paramyosin Trypsin Resistant Core 1 (α-PTC-1) and β-Paramyosin Trypsin Resistant Core 1 (β-PTC-1).* The trypsin-resistant cores of α- and β-paramyosins were formed by trypsin digestion of the paramyosin and purified by the procedure described by Cowgill (1972).

General Procedures

1. *Dansylation of the Proteins for Determination of N-Terminal Residues.* The procedure of Gros and Labouesse (1969) was followed for dansylation and hydrolysis of the proteins and separation of the dansylated amino acids by TLC.

2. *Treatment of the Proteins with Leucine Aminopeptidase.* The reduced forms of both α- and β-paramyosins were subjected to digestion by the peptidase according to the directions of Smith and Hill (1960) and the amount of amino acid liberated was followed by assay with fluorescamine (Udenfriend et al., 1972). Morpholinopropanesulfonic acid was employed as buffer because Tris buffer was found to react with fluorescamine to yield a highly fluorescent product. The peptidase was shown to be active in the former buffer by its ability to hydrolyze the dipeptide Leu-Trp.

3. *Reaction of the Proteins with Carboxypeptidase A.* Digestions of α-paramyosin, β-paramyosin, α-CCF-2, and β-CCF-2 were performed at pH 8.5 in 0.3 M KHCO$_3$ at 27 °C for 30 min by the general procedure recommended by Light (1972). The paramyosins were at 6 mg/ml; the α-CCF-2 and β-CCF-2 were at 2 mg/ml. The possibility of self-digestion by the peptidase was monitored and found to be insignificant. The reaction products were deproteinized with perchloric acid and then desalted by neutralization of the solution with KOH to form the insoluble KClO$_4$ salt. After centrifugation to remove KClO$_4$, the quantity of amino acids liberated was estimated by the fluorescamine assay as above. After evaporation to dryness, the amino acids were resolubilized and identified by comparison with standard amino acids on TLC.

4. *Determination of Sulfhydryl Groups.* The amount of cysteinyl sulfhydryl groups reactive with Nbs$_2$ was determined by the procedure of Ellman (1959) as modified by Cowgill (1974). The molar extinction coefficient of 2-nitro-5-thiobenzoate released was taken as ϵ_{412} 13 600. The total cysteine content of α- and β-paramyosins, regardless of their extent of
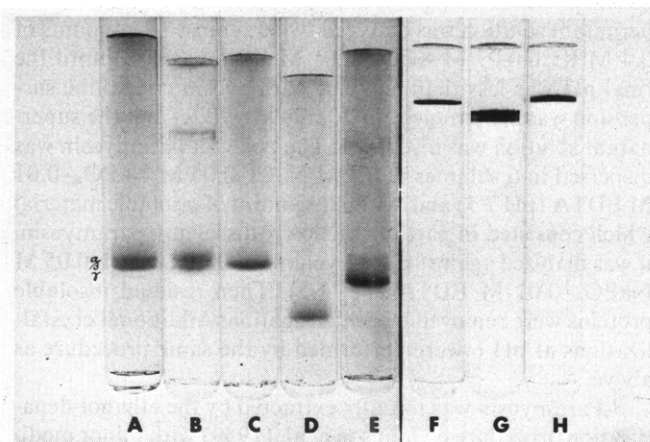
FIGURE 1: Stained protein bands following gel electrophoresis. Gels A–E were done by dodecyl sulfate gel electrophoresis of α- and β-paramyosins in 5% acrylamide–0.135% bisacrylamide gels: (A) α- and β-paramyosins in coelectrophoresis (a component that is thought to be γ-paramyosin is seen as a faint band below the β-paramyosin band); (B) α- and β-paramyosins plus rabbit myosin in coelectrophoresis; (C) α-paramyosin alone; (D) β-paramyosin alone; (E) β-paramyosin by the selective-extraction procedure and β-paramyosin by the ethanol-denaturation procedure in coelectrophoresis (a faint band of γ-paramyosin contamination can be seen). The gels were run 16 h at 7 mA/gel except D was run for 18 h. The buffer system of 0.1% dodecyl sulfate–0.1 M NaPO₄ (pH 7.2) was continuously circulated between top and bottom reservoirs. Gels F–H were done by electrophoresis of α- and β-paramyosins at pH 2.3 and 200 V for 4 h: (F) β-paramyosin; (G) α-paramyosin as the upper band upon coelectrophoresis with β-paramyosin as the lower band; (H) α-paramyosin alone. These last three gels were not run in the same experiment but the same preparations of α- and β-paramyosins were used for coelectrophoresis and for electrophoresis of the individual samples.

oxidation, was determined by the method of Butterworth et al. (1967) as modified by Cowgill (1974).

5. Specific Chemical Cleavage of the Polypeptide Chain of Paramyosin at Sites of Cysteine Residues. This cleavage reaction was applied to β-paramyosin by Cowgill (1974, 1975) by the two-step procedure of Jacobson et al. (1973) with minor modifications. The same method has now been applied to α-paramyosin.

6. Acrylamide Gel Electrophoresis. The method of Davis (1965) was followed except for the elimination of the stacking gel as suggested by Mitchell (1967). Electrophoresis was done both at pH 2.3 and pH 9.5 on gels of 7 mm diameter and 7.8 cm length. Gels were scanned with a Gilford spectrophotometer that was equipped with a densitometer attachment. The sodium dodecyl sulfate gel electrophoresis procedure was that of Weber and Osborn (1969) but modified as follows. Mercaptoethanol was replaced with dithioerythritol. Sample preparation consisted of 0.1 ml of a protein at 3 mg/ml incubated with 0.3 ml of 2% sodium dodecyl sulfate plus 1% dithioerythritol at pH 7 for 2 h at 37 °C. Proteolytic enzymes used as molecular weight standards were rocked for 2 min in a 100 °C bath before incubation at 37 °C for 2 h and, after this treatment, no proteolytic degradation was observed. Samples were not combined for coelectrophoresis until just before application to the gel and a 20-μl sample that contained 5 μg of each protein was applied to the gel. For electrophoresis, a system was used for continuous circulation of buffer between upper and lower reservoirs except in the first 15 min of electrophoresis. The gels were run for 12–18 h at 7 mA per gel. The electrophoresis buffer was 0.1% sodium dodecyl sulfate–0.1 M NaPO₄ (pH 7.2). The Coomassie blue R250 background stain in the gels after electrophoretic destaining was cleared

completely by soaking the gels overnight in a solution of water–methanol–acetic acid (60:30:10 parts by volume).

7. Solubility tests of paramyosin were done at 4 °C and pH 7.5, with variation of ionic strength. Paramyosin at a concentration of 3–4 mg/ml was equilibrated by dialysis against 0.4 M NaCl–0.05 M NaPO₄–0.01 M EDTA (pH 7.5) at 4 °C. Then aliquots were equilibrated in a rocking dialyzer for 18 h at 4 °C against solutions of 0.05 M NaPO₄–0.01 M EDTA (pH 7.5) that contained variable amounts of NaCl to achieve the desired ionic strength. For ionic strengths below 0.2, it was necessary to lower the concentration of phosphate buffer as well as omit NaCl. After equilibration, the contents of each dialysis bag was centrifuged 20 min at 5900g at 4 °C. The absorbance of the supernatant solution was measured at 276 nm and the solubility in mg/ml was computed from the values $\epsilon_{276}^{1\%}$ 2.96 for α-paramyosin and 3.05 for β-paramyosin.

Results

A. Extraction of α-Paramyosin and Its Partially Degraded Form β-Paramyosin. α-Paramyosin, the form of paramyosin that apparently exists in adductor muscle, could be extracted in the presence of EDTA to prevent proteolysis. In the absence of EDTA the partially degraded form β-paramyosin was obtained as well as a small amount of the more extensively degraded γ-paramyosin form (Stafford and Yphantis, 1972). If these extractions were by the procedure of Johnson et al. (1959) that includes an ethanol-denaturation step, the yield of α-paramyosin was low (5 mg/g of muscle) compared with the yield for β-paramyosin of about 20 mg/g of muscle. The cause for the low yield of α-paramyosin was traced to the difficulty of resolubilizing α-paramyosin from the proteins precipitated by the ethanol. The selective-extraction procedure described in the Materials and Methods section circumvents the ethanol-denaturation step and yields of 15–25 mg/g of muscle were obtained at the stage of the third crystallization. α-Paramyosin was homogeneous by sodium dodecyl sulfate gel electrophoresis but β-paramyosin extracted by the selective-extraction procedure was accompanied by a small amount of γ-paramyosin. β-Paramyosin extracted by the ethanol-extraction procedure was free from γ-paramyosin but contained a small amount of α-paramyosin.

B. Molecular Weight of α-Paramyosin and Its Partially Degraded Forms. Under the conditions described for sodium dodecyl sulfate gel electrophoresis of paramyosin, it was possible to separate the single chains of α-, β-, and γ-paramyosins and the results are shown in Figure 1. It may be noted that β-paramyosin extracted by the ethanol-denaturation procedure and β-paramyosin extracted by selective-extraction procedure migrated together upon coelectrophoresis in Figure 1E and therefore appear to be identical. The single chain of β-paramyosin was found to be nonresolvable from rabbit muscle phosphorylase A (100 000) even after 18 h of coelectrophoresis; therefore the single chain of β-paramyosin was assigned a value of 100 000. The molecular weights of α- and γ-paramyosin single chains were determined with respect to those of rabbit myosin (200 000) and β-paramyosin (or phosphorylase A) as standards, and the molecular weights of the double chain molecules were determined to be 207 000 for α-paramyosin, 200 000 daltons for β-paramyosin, and 193 000 for γ-paramyosin. The α- and β-paramyosins could also be distinguished by gel electrophoresis at pH 2.3 as shown in Figure 1.

C. Solubility of α-Paramyosin and β-Paramyosin. The α- and β-paramyosins could be distinguished by solubility differences as well as by differences in electrophoretic mobility.
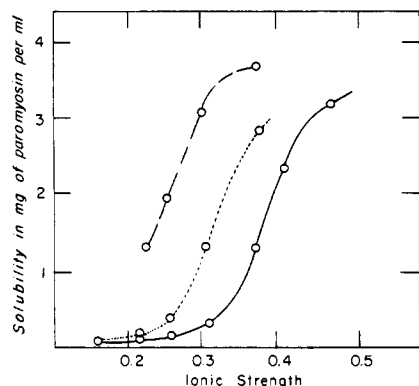
FIGURE 2: Solubility curves of α-paramyosin (—), β-paramyosin (——), and a slightly degraded form of α-paramyosin described in the text (- - -). Experiments were at 4 °C in 0.01 M EDTA–0.05 M NaPO₄ at pH 7.5 plus variable amounts of NaCl to yield the indicated total ionic strengths.
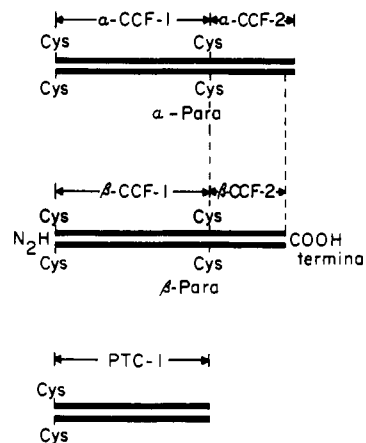

FIGURE 3: Schematic representation of α- and β-paramyosins with emphasis upon location of Cys residues and the origins of segments obtained by chemical or trypsin cleavage reactions. The two α-helical polypeptide chains are represented by the two solid lines.

The solubility curves are shown in Figure 2. α-Paramyosin was found to be much less soluble than β-paramyosin at lower ionic strengths and this is in agreement with the observations of Merrick and Johnson (1974). Below an ionic strength of 0.2, the β-paramyosin aggregated into a gel and the absorbance values became erratic because of light scatter. The intermediate curve is for a degraded form of paramyosin that can form when α-paramyosin is stored for a month or more at 4 °C and neutral pH even in the presence of EDTA. This form was less degraded than β-paramyosin by the criterion of sodium dodecyl sulfate gel electrophoresis for it occurred between α- and β-paramyosins in experiments such as the one in Figure 1A. Apparently some of the tissue protease continues to contaminate the α-paramyosin even after several crystallizations and can slowly act even in the presence of EDTA. Investigators should be aware of this for the slightly degraded paramyosin is difficult to distinguish from α-paramyosin by gel electrophoresis and yet may profoundly affect properties such as solubility as in Figure 2. The residual protease can be removed by recrystallization four or more times at pH 6.

D. Determination of Structural Differences between α-Paramyosin and β-Paramyosin. The molecular weights given above indicate that α-paramyosin is larger than β-paramyosin by about 7000, and it is presumed that a segment of α-paramyosin was removed by a tissue protease under the conditions that led to isolation of β-paramyosin. The location of this segment was established by several approaches.

(1) Comparison of the N-Terminal Amino Acids of α- and β-Paramyosins. In earlier studies of β-paramyosin the only N-terminal residue found was Cys (Cowgill, 1972). Because two Cys residues occur in that region and form an interchain disulfide bond (Cowgill, 1974), it is possible that both peptide chains of the molecule terminate with Cys residues. In the present experiments, the dansylation procedure for determination of N-terminal amino acids was employed again and both α- and β-paramyosins were compared. Both forms of paramyosin gave the same dansylated amino acids and once again the N-terminal amino acid would appear to be Cys by the same criteria discussed in the earlier paper.

No amino acids were liberated from either α- or β-paramyosin by leucine aminopeptidase under the digestion conditions described in the Materials and Methods section. This is consistent with other evidence that the N-terminal region of β-paramyosin is exceptionally stable in the α-helical conformation and indicates that the N-terminus of α-paramyosin also is exceptionally stable.

(2) Comparison of the C-Terminal Amino Acids of α- and β-Paramyosins. Under the conditions described in the Materials and Methods section, carboxypeptidase released one amino acid plus traces of several others from each form of paramyosin. The major amino acid liberated from α-paramyosin was valine and that from β-paramyosin was aspartic acid. This difference at the C-terminal end plus the apparently identical N-terminal ends of α- and β-paramyosins indicate that the segment was lost from the C-terminus upon conversion of α- to β-paramyosin. Confirmatory evidence was obtained by cleavage of the paramyosin at Cys residues.

(3) Specific Chemical Cleavage of the Polypeptide Chain of Paramyosin at Sites of Cys Residues. Jacobson et al. (1973) have described a chemical procedure for specific cleavage of a polypeptide chain on the amino terminal side of Cys residues, and the method has been successfully employed in this laboratory for location of Cys residues in β-paramyosin (Cowgill, 1974). Based on the proposed structure of β-paramyosin shown in Figure 3, it should be possible to gain same information about the location of the additional segment in α-paramyosin by comparison of the sizes of CCF-1 and CCF-2 cleavage fragments from α- and β-paramyosins. For this comparison to be valid it was necessary to demonstrate that both α- and β-paramyosins have the same number of Cys residues. Assays of the purified proteins for the number of reduced sulfhydryl groups gave values in the range 1.0–1.5 SH/molecule for α-paramyosin and 2.5–3.0 SH/molecule for β-paramyosin. However, some oxidation of Cys residues occurred during the isolation procedures and assays for total Cys residues established that both α-paramyosin and β-paramyosin had 4 Cys/molecule. The α- and β-paramyosins were subjected to the chemical cleavage of their polypeptide chains at the sites of their Cys residues and the products were analyzed by electrophoresis as shown in Figure 4. The products from β-paramyosin revealed the three major bands of paramyosin, β-CCF-1, and β-CCF-2, as reported earlier by Cowgill (1974, 1975). Other minor bands are probably the products of side reactions which are known to occur (Degani and Patchornik, 1974). α-Paramyosin, after undergoing the cleavage reaction, revealed an almost identical band pattern on electrophoresis, except that the segment we term α-CCF-2 appeared to have a slightly lower mobility than β-CCF-2. This was confirmed by coelectrophoresis of the products from α- and β-paramyosins as shown in Figure 4C. α-CCF-1 and β-CCF-1 were not
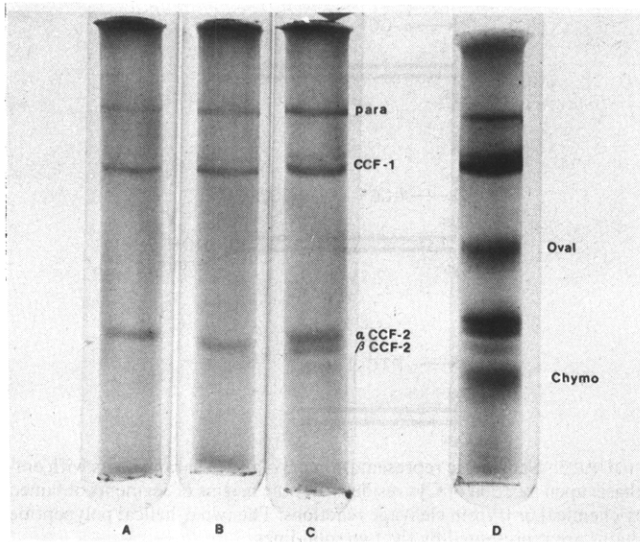
FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of unfractionated Cys-cleavage products of α- and β-paramyosins. (A) Products from α-paramyosin; (B) products from β-paramyosin; (C) coelectrophoresis of products from α- and β-paramyosins; (D) coelectrophoresis of Cys-cleavage products of α- and β-paramyosins and the molecular weight standards of ovalbumin and α-chymotrypsinogen A. Note separation of α-CCF-2 and β-CCF-2 bands in C and D. In D, the band between β-CCF-2 and α-chymotrypsinogen A was attributed to γ-CCF-2 from the small amount of γ-paramyosin present in the β-paramyosin used. The gels were 10% acrylamide–0.2% bisacrylamide, run 12 h at 8.5 mA/gel. D was from a separate experiment.

separable under any conditions of gel electrophoresis described in the Materials and Methods section. The molecular weights of cleavage fragments were determined from sodium dodecyl sulfate gel electrophoresis experiments.

From these data the molecular weights of the CCF segments could be calculated on the assumption that the observed values were for the single polypeptide chains and that the CCF segments still contain two α-helical polypeptide chains. (The circular dichroism values reported earlier (Cowgill, 1975) certainly indicate that β-CCF-1 and β-CCF-2 remain helical.) The molecular weights are 137 600 for both α- and β-CCF-1, 64 000 for α-CCF-2 and 61 600 for β-CCF-2. The molecular weight difference which was consistently observed between α-CCF-2 and β-CCF-2 indicated that α-CCF-2 was about 2400 daltons heavier than β-CCF-2. This further supports the finding that the extra segment is at the C-terminal end of α-paramyosin. There appears to be a discrepancy in these molecular weight differences because α- and β-paramyosins differ by 7000, but α-CCF-2 and β-CCF-2 apparently differ by only 2400. If all of the segment removed in degradation of α-paramyosin to β-paramyosin was at the C-terminal end and if the structural scheme in Figure 3 is correct, then α- and β-CCF-2 also should differ by 7000. However, the discrepancy probably is due to technical limitations in attempting to compare to this degree of accuracy the molecular weights of two molecules that differ only 3.5% from one another and also to differences that arise from attempting to compare values obtained at three different conditions of electrophoresis. (The large paramyosin chains were electrophoresed under conditions described in Figure 1; CCF-1 segments were compared with protein standards under the same conditions, except that electrophoresis was limited to 8 h; the smaller CCF-2 segments were compared with protein standards under the conditions described in Figure 4.)

The consistency of these results and the validity of the

structure proposed in Figure 3 were further established by determining the C-terminal amino acids of α-CCF-2 and β-CCF-2 after isolation of these segments as described by Cowgill (1975). Digestion by carboxypeptidase showed the same liberated amino acids for α-CCF-2 as for α-paramyosin and the ones liberated from β-CCF-2 were the same as those reported above for β-paramyosin.

E. Comparison of the Susceptibility of α-Paramyosin and β-Paramyosin to Digestion by Trypsin and Pepsin. It has been reported by Cowgill (1972) and by Halsey and Harrington (1973) that only the C-terminal one-third of the β-paramyosin molecule was digested by trypsin and that a trypsin-resistant core (PTC-1) accumulated. α-Paramyosin was found to produce the same trypsin resistant core as judged by gel electrophoresis at pH 2.3 and 9.5. The α-PTC-1 and β-PTC-1 products were shown to be identical also by the fact that neither segment was digested further by pepsin at pH 2. This resistance to pepsin had been reported earlier for β-PTC-1 by Cowgill (1972). These results are consistent with the earlier studies that led to the proposed structure shown in Figure 3 for paramyosin and bolster our confidence in this proposal.

Discussion

Studies on α-paramyosin have been hampered by the very low yield when the protein was extracted by the ethanol-denaturation procedure. A yield that is comparable with that obtained for β-paramyosin in the absence of EDTA would be desirable also to ensure that α-paramyosin does not constitute a minor fraction of the total paramyosin in the thick filament. The development of the selective-extraction procedure has accomplished these aims by circumventing the ethanol precipitation which appears to be the step where α-paramyosin is lost. The solubilities of the different forms of paramyosin at neutral pH are different as the curves in Figure 2 show. These solubility differences permitted us to detect a slightly degraded form of paramyosin which is difficult to distinguish from α- or β-paramyosin by gel electrophoresis but which apparently is of an intermediate size. The α- and β-paramyosins can be distinguished also by gel electrophoresis at pH 2.3 as shown in Figure 1 and by sodium dodecyl sulfate gel electrophoresis which indeed was the first means of detecting α-paramyosin by Stafford and Yphantis (1972). The technique employed in this laboratory for sodium dodecyl sulfate gel electrophoresis of these high molecular weight proteins permits direct coelectrophoresis with high resolution. This technique is preferable to the split-gel technique for detecting small differences in molecular weight as in the case of α- and β-paramyosins. In our experience, the small variations in the gel surfaces of the two sides of the septum inserted into the gel in the split-gel technique were sufficient to produce significant artifacts. The molecular weights obtained for α-, β-, and γ-paramyosins were 207 000, 200 000, and 193 000, respectively, which differ only slightly from the corresponding values of 210 000, 200 000, and 188 000 reported by Stafford and Yphantis (1972) using the split-gel technique.

Because α-paramyosin may be the native form of the molecule as it occurs in the thick filament and yet β-paramyosin is the form on which most of the past studies have been made, it has become important to know the location of the small segment which was lost from α-paramyosin upon conversion to β-paramyosin. Evidence for the location of the extra segment came from the N-terminal and C-terminal amino acid determinations of the purified α- and β-paramyosins. As shown in the Results section, the N-terminal ends of α- and β-paramyosins appear to be the same by both the dansylation labeling

of N-terminal amino acids and by the nonsusceptibility to attack by leucine aminopeptidase. However, differences were found at the C-terminal ends because attack by carboxypeptidase released aspartic acid from β-paramyosin, but valine was found to occupy the C-terminal position of α-paramyosin. Thus it is apparent that the extra segment in α-paramyosin which is absent in β-paramyosin is located at the C-terminal end. Confirmation of this conclusion came from molecular weight comparisons of the segments of α- and β-paramyosins obtained by specific chemical cleavage at the sites of the Cys residues (Figure 3). The segments α-CCF-1 and β-CCF-1 behaved the same on sodium dodecyl sulfate gel electrophoresis whereas the α-CCF-2 and β-CCF-2 segments were separable (Figure 4) and this behavior indicates that α-CCF-2 was larger than β-CCF-2.

It has been shown by Cowgill (1975) that the solubility characteristics of β-paramyosin at low ionic strength and neutral pH are determined by the C-terminal region that corresponds to the β-CCF-2 portion of the molecule. It seems likely that the same will be true for α-paramyosin. That is, the CCF-1 region does not possess a great tendency for self-aggregation at neutral pH as denoted by its high solubility independent of ionic strength. The α-CCF-2 region, by analogy with β-CCF-2, would account for the strong self-aggregation of α-paramyosin and it appears quite likely that the small additional segment on α-paramyosin causes the marked differences in solubility seen in Figure 2 by further increasing the tendency toward self-aggregation.

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